PATENT

Attorney Docket No. 012418-001820

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
VANITHA RAMAKRISHNAN, et al.	Examiner: P. Gambel
Serial No.: 08/258,283	Art Unit: 1806
Filed: June 10, 1994	
For: INHIBITORY IMMUNOGLOBULIN POLYPEPTIDES TO HUMAN PDGF BETA RECEPTOR	DECLARATION OF DR. NEILL GIESE UNDER 37 C.F.R. 1.132

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Neill Giese, declare as follows:

1. I am presently employed as a Senior Scientist at COR Therapeutics, Inc., in South San Francisco California, the assignee of record in the above-captioned patent application. I received a Ph.D. from the University of Arizona in 1984. I have over ten years of experience working in the field of growth factors and tyrosine kinase receptors, including fibroblast growth factor receptor (FGFR) and platelet derived growth factor receptor (PDGFR). This work has focused on mechanisms responsible for PDGFR binding, dimerization and autophosphorylation. For the past six years, I have been the Group Leader for the Growth Factor Inhibitor program at COR Therapeutics. This has involved identification of the role of FGF and PDGF in cardiovascular disease and development of specific antagonists of these factors for the prevention of restenosis following coronary angioplasty. A copy of my Curriculum Vitae is attached hereto.

2. I have followed the prosecution history of the above-captioned application and I have read the Office Action mailed December 23, 1994, in this application. I understand that the

Examiner has rejected several of the pending claims as allegedly obvious over Williams, et al., Science 243:1564-1570 (1992) ("Williams I"), Published European Patent Application No. 0 327 369, to Williams, et al. ("Williams II"), and U.S. Patent No. 5,268,358, to Fretto, et al. ("Fretto"), in view of Kawahara, et al., Biochem. Biophys. Res. Commun. 147:839-845 (1987) ("Kawahara"). I further understand that the Examiner has relied on the Kawahara reference's discussion of the alleged PDGF-receptor specific antibodies as having suggested the ability to derive PDGF-receptor specific antibodies having the similar functions as the claimed antibodies.

3. I respectfully disagree with the Examiner's assertion that the combination of the teachings of the primary references, Williams I and II and Fretto, in view of the teachings of the Kawahara reference, would have taught or suggested the antibodies of the present invention. In particular, I disagree with the Examiner's assertions that the Kawahara reference would have suggested to one of ordinary skill in the art, the ability to produce antibodies specific for PDGFR, and which antibodies would possess the same functions as those of the present invention. I offer the following reasons in support of my conclusion:

a. The immunization protocols used in the Kawahara reference would not have taught one of ordinary skill in the art the ability to produce antibodies specific for βPDGFR, let alone antibodies specific for the second Ig-like domain of human βPDGFR, as are claimed in the above-captioned application. Specifically, the Balb/C mice used in Kawahara to produce the antibodies described therein, were first immunized with the mouse Swiss 3T3 cell line designated NR6. These whole cells contain very large numbers of potentially antigenic proteins, of which PDGFR would represent only a small fraction. The protein preparation used to boost these mice was prepared by fractionating cell membrane proteins from NR6 cells using wheat germ affinity chromatography. Because wheat germ generally binds all glycosylated proteins, the preparation used to boost the animals would be expected to contain large numbers of different

proteins from the NR6 cells. This "purification" would result in only a marginal enrichment for PDGFR (up to 10-fold) which would still account for only a small percentage of the total protein. Therefore, immunization with wheat germ purified proteins isolated from cell membranes would be expected to generate antibodies against many different proteins. Kawahara screened his hybridomas against the overall wheat germ preparation which would not selectively identify monoclonal antibodies against PDGFR, but would identify monoclonal antibodies against any protein in the preparation. Since only 14 monoclonal antibodies were identified, the probability of the C3.1 antibody being against PDGFR is very low.

In addition to the above, at the time of the Kawahara reference, only a single PDGFR was known, later termed the βPDGFR. However, NR6 cells are now known to contain both the α- and βPDGFR isoforms. Kawahara did not teach the existence of both isoforms of PDGFR, and certainly did not teach antibodies specific for one particular PDGFR isoform. Based upon all of the above-described aspects of the Kawahara immunization protocols, one of ordinary skill in the art would not have been led to believe that the antibodies described therein would be specific for PDGFR, generally, or human β-PDGFR, specifically.

Finally, the 2A1E2 antibody claimed in the above-captioned application (expressed by the cell line ATCC HB10938), was raised against purified human β-PDGFR and reacts only with human or primate receptors, and not with that of lower species. Antibodies raised against mouse receptors as described in Kawahara on the other hand would be far less likely to cross-react with a human receptor.

b. In order to demonstrate that the C3.1 monoclonal antibody inhibited PDGFR mediated mitogenic responses, its ability to block PDGF induced [^{3}H]thymidine incorporation into NR6 cells was tested. C3.1 showed no significant inhibition of PDGF induced mitogenesis until antibody concentrations of > 1.3 μM were used. When monoclonal antibody concentrations this high are used in mitogenic assays, it is not uncommon to observe nonspecific inhibitory effects and no experiments were done by

Kawahara to demonstrate specificity. For example, the effect of C3.1 on FGF or serum induced mitogenic response in NR6 cells should have been tested. Therefore, Kawahara's data would not have led the skilled artisan to believe that this reference taught an antibody specific for PDGFR.

c. As evidence that C3.1 recognized PDGFR, Kawahara demonstrated that this antibody immunoprecipitated a phosphorylated protein, present in the wheat germ preparation, that migrated in the same molecular weight size range as PDGFR when analyzed by SDS-PAGE. Although a 180 kDa component which corresponds to the approximate size of PDGFR was immunoprecipitated, this data would not be recognized by one skilled in the art as having demonstrated that the C3.1 antibody is specific for human βPDGFR. In order to phosphorylate proteins so they could be detected on polyacrylamide gels following immunoprecipitation, the wheat germ preparation was first radiolabeled by incubation with [^{32}P]ATP. Under these conditions, very little if any incorporation of phosphate into the PDGFR would be expected, because PDGF binding is required for PDGFR autophosphorylation and no PDGF was included in the incubations. Therefore, it is likely that the 180 kDa protein detected in this experiment represents a membrane component which was phosphorylated by any of a number of kinases which could have been present in the wheat germ preparation.

d. To demonstrate that C3.1 and PDGF recognize overlapping epitopes, the ability of C3.1 to immunoprecipitate PDGFR when bound to PDGF was examined. Preincubation of the ^{32}P-labeled wheat germ preparation with PDGF caused only a slight decrease in the 180 kDa band which corresponds to the size of PDGFR. A greater loss of detection of proteins in the 60-90 kDa range was observed which would not be the PDGFR. Also, a PDGF concentration of 1000 nM was required to see this effect which is 1000 fold higher than the concentration required to reach saturation binding of the PDGFR under these conditions. Because of this, it is very unlikely that the decrease in the immunoprecipitation of any protein was due to a specific interaction between PDGF and its receptor. More importantly,

preincubation with FGF caused a comparable decrease in the immunoprecipitation of phosphorylated proteins by C3.1. Furthermore, the standard procedure to determine if C3.1 and PDGF compete with each other for binding to PDGFR would be to examine the effects of the antibody on ^{125}I-PDGF binding to its receptor on intact cells, which was not done by Kawahara.

4. Based upon all of the reasons provided, it is my conclusion that the Kawahara reference would not have taught one of ordinary skill in the art how to produce antibodies that are specific for PDGFR, generally, and more specifically, this reference would not have taught the ability to produce antibodies specific for the second Ig-like domain of human βPDGFR.

5. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under § 1001 of Title 18 of the United States Code, and that such willful false statement may jeopardize the validity of the application or any patent issuing thereon.

Date: 9/29/95

NEILL GIESE, Ph.D.

COR Therapeutics, Inc.
256 E. Grand Avenue
South San Francisco, CA 94080

Attachments:

Curriculum Vitae

12418\18Giese.DEC

I hereby certify that this correspondence is deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, BOX AF, Washington, D.C. 20231 on

4-10-96

By Christina Borgeno

PATENT
Attorney Docket No. 12418-18-2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Vanitha Ramakrishnan, et al.	Examiner: P. Gambel
Serial No.: 08/258,283	Art Unit: 1806
Filed: June 10, 1994	SECOND DECLARATION OF DR. NEILL GIESE UNDER 37 C.F.R. 1.132
For: INHIBITORY IMMUNOGLOBULIN POLYPEPTIDES TO HUMAN PDGF BETA RECEPTOR	

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Neill Giese, declare as follows:

1. I am presently employed as a Senior Scientist at COR Therapeutics, Inc. ("COR"), in South San Francisco, California, the assignee of record in the above-referenced patent application. I received a Ph.D. from the University of Arizona in 1984. I have over ten years of experience working in the field of growth factors and tyrosine kinase receptors, including fibroblast growth factor ("FGF") receptor and platelet derived growth factor ("PDGF") receptor. This work has focused on mechanisms responsible for PDGF receptor binding, dimerization and autophosphorylation. For the past six years, I have been the Group Leader for the Growth Factor Inhibitor program at COR. This has involved identification of the role of FGF and PDGF in cardiovascular disease and development of specific antagonists of these factors for the prevention of restenosis following coronary angioplasty. A copy of my Curriculum Vitae is attached hereto, as Attachment A.

2. The above-referenced patent application describes immunoglobulin polypeptides that block binding of PDGF to the beta PDGF receptor. The immunoglobulin polypeptide

illustrated in the examples is referred to as the 2A1E2 monoclonal antibody (the "2A1E2 MAb").

3. In my position as Group Leader I am involved with reviewing the results of scientific research that is performed at academic institutions on behalf of COR, including the work performed by Dr. Steve Hanson at Emory University, who has been a collaborator and scientific advisor to COR for many years in the area of thrombosis and restenosis.

(a) Dr. Hanson has developed a baboon model for the study of restenosis, which is described in Hypertension 18(4): II-70 to II-76 (October, 1991), a copy of which is attached hereto as Attachment B. In this article, Dr. Hanson indicates that the baboon was chosen as an animal model because its vascular anatomy and hemostatic mechanisms are similar to those in humans.

(b) At COR's request, Dr. Hanson used this model to evaluate the 2A1E2 MAb (the "Study"). As described in detail below, short term administration of the 2A1E2 MAb efficiently inhibited neointima formation in the baboon following carotid endarterectomy and femoral artery balloon injury. This result indicates that anti-PDGF receptor antibodies, such as the 2A1E2 MAb, have therapeutic potential for the prevention of PDGF-mediated diseases such as restenosis.

4. The Study involved the following procedure:

(a) Juvenile male baboons aged 2-3 years and weighing 12-16 kg were used in the Study. All animals were quarantined and observed to be disease free for at least 90 days before use during which time they were maintained on a normal diet. The treated and control groups were each made up of 7-10 baboons. The experimental arterial injury in the treated and control groups was produced by two different methods, simulating therapeutic procedures performed in humans, namely carotid artery endarterectomy and femoral balloon catheter vessel dilation, which were performed concurrently.

(b) The 2A1E2 MAb was given by intravenous bolus (40 mg) immediately before injury and then once every 24 hours at 20 mg/dose for an additional 5 days. In order to determine plasma levels of the 2A1E2 MAb, samples were obtained just prior to and just after each antibody administration. The plasma concentrations of the 2A1E2 MAb,

as determined by a standard ELISA, were maintained over the first 6 days in the range of 125-150 nM at peak and the trough concentrations ranged from 75-100 nM. These concentrations are >100-fold higher than those required to block beta PDGF receptor function *in vitro*, as is shown in the above-referenced patent application.

(c) Animals were sacrificed at 30 days after injury, vessels were fixed in 2% paraformaldehyde, embedded in paraffin and cross sectioned. Morphometric measurements were performed using a Zeiss photoscope coupled with a computer-assisted display system. The response to femoral balloon injury was represented as % neointima which is determined by the formula: neointimal area/neointimal area + medial area x 100 = % neointima. The response to carotid endarterectomy was presented as neointima area (mm^2) without consideration of medial areas because the procedure tends to remove variable amounts of the media.

(d) Treatment of baboons for 6 days with the 2A1E2 MAb inhibited the vascular response to injury induced by carotid artery endarterectomy and femoral artery ballooning. Morphometric analysis of the carotid arteries revealed a 30% reduction in neointimal area in the 2A1E2 MAb-treated group, as shown in Figure 1 of Attachment C. This result is significant, especially in view of the fact that endarterectomy is a severe injury that produces a high level of platelet deposition and thrombus formation which provides a strong stimulus for lesion formation. Ballooning of the femoral arteries produces a less severe injury and in this case, treatment with the 2A1E2 MAb resulted in an even greater level of reduction in lesion formation. As shown in Figure 2 of Attachment C, the neointimal area for the control group of 14.7 mm^2 was lowered to 8.2 mm^2 by treatment with the 2A1E2 MAb, which represents a 45% reduction. This is the highest level of inhibition observed for any agent tested in this model. Low molecular weight heparin gave a comparable level of inhibition when administered continuously for 30 days, but demonstrated no inhibition when given for a shorter duration as was the 2A1E2 MAb.

(e) The Study demonstrates that treatment with the 2A1E2 MAb for one week after vascular injury in the baboon causes a significant reduction in lesion formation when measured one month later. Such results strongly implicate PDGF as a key mediator of the biological response immediately following vascular injury, whether induced by vasodilatation or endarterectomy. These findings are of particular interest

because they demonstrate the role of PDGF in mediating neointimal formation in a primate model of restenosis. This model is considered to be the most relevant to clinical restenosis. As indicated by Dr. Hanson in Hypertension, *supra* at page II-70,

> "Baboons were chosen as the experimental animal because of their primate vascular anatomy and because the primate hemostatic mechanism and renin-angiotensin system are similar to those in humans (citations omitted)".

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing hereon.

2/7/96
Date

Neill Giese
NEILL GIESE, Ph.D.

COR Therapeutics, Inc.
256 Grand Avenue
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CURRICULUM VITAE

Name: Neill A. Giese

Citizenship: U.S.A.

Education and Training:

1971-1973	Pre-pharmacy, University of Oregon, Eugene, Oregon
1973-1976	B.S., College of Pharmacy, Oregon State University, Corvallis, Oregon
1978-1980	M.S., Oregon State University College of Pharmacy, Dept. of Pharmacology (Pharmacology/Toxicology)
1980-1984	Ph.D., University of Arizona College of Medicine, Dept. of Pharmacology

Brief Chronology of Employment:

1976-1978	Staff Pharmacist, St. Vincent Hospital, Portland, Oregon
1984-1987	Guest Researcher, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland
1987-1989	IRTA Fellow, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland
1989-1993	Senior Scientist, Growth Factor Program Leader, COR Therapeutics, Inc., South San Francisco, California
1993-present	Senior Scientist II, Growth Factor Program Leader, COR Therapeutics, Inc. South San Francisco, California

Honors and Awards:

1981-1984	USPHS-NCI Predoctoral Fellowship
1984-1989	USPHS-HCI Postdoctoral Fellowship

Societies:

1978	Rho Chi: Pharmacy Honor Society

Research Interests:

-Molecular regulation of vascular cell growth
-Growth factor structure and function
-Mechanisms of growth factor receptor activation and signal transduction
-Intracellular signaling mechanisms

Present Address: 1507 Dolores St.
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ATTACHMENT A

Patents

1. Wolf, D. Tomlinson, J. E., Fretto, L. J., Giese, N., Escobedo, J. A. and Williams, L. T. Domains of the Extracellualr Region of the Human PDGF Receptor Polypeptides. Filed 1/31/91 (U.S.) Serial No. 07/650,793.

2. Giese, N. Receptor Function Assays. Filed 7/29/93 (U.S.) Serial No. 08/099,850.

3. Giese, N. and Lokker, N. Methods and Composition for Inhibiting Protein Kinases. Filed 10/28/94 (U.S.) Serial No. 08/332,597.

4. Giese. N. and Lokker, N. Pharmaceutical Pyrazole Compositions Useful as Inhibitors of Protein Kinases. Filed 11/10/94 (U.S.) Serial No. 08/337,630.

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Neill A. Giese

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6 Robbins, K.C., King, C.R., Giese, N.A., Leal, F., Igarashi, H., and Aaronson, S.A. : Involvement of oncogene-coded growth factors in the neoplastic process. In Papas, T.S. and Vande Woude, G.G. (Eds): Gene Amplification and Analysis. Oncogenes and Growth Factors. New York, Elsevier, pp. 161-176, 1986.

7 Giese, N.A., Robbins, K.C., and Aaronson, S.A. : The role of individual cysteine residues in the structure and function of the v-sis gene product. Science. 326:1315-1318, 1987.

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9. Giese, N., May-Siroff, M., LaRochelle, W.J. and Aaronson, S.A. : Localization of a region within the v-sis/PDGF-B protein required for receptor activation and neutralizing monoclonal antibody recognition. Mol. & Cell. Biol. 10:5496-5501, 1990.

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Effects of Angiotensin Converting Enzyme Inhibition With Cilazapril on Intimal Hyperplasia in Injured Arteries and Vascular Grafts in the Baboon

Stephen R. Hanson, Jerry S. Powell, Thomas Dodson, Alan Lumsden, Andrew B. Kelly, Johanna S. Anderson, Alexander W. Clowes, and Laurence A. Harker

To determine the importance of angiotensin converting enzyme (ACE) activity in the development of arterial proliferative lesions in a primate model, the response to vascular injury was studied in five baboons treated with oral cilazapril (20 mg/kg/day) and in five untreated control animals. Each animal underwent three procedures: 1) carotid artery endarterectomy, 2) balloon catheter deendothelialization of the superficial femoral artery, and 3) surgical placement of bilateral aorto-iliac expanded polytetrafluoroethylene (Gore-Tex) vascular grafts. Cilazapril therapy was initiated 1 week preoperatively and continued throughout the study interval. At 1 and 3 weeks postoperatively, plasma ACE activity was inhibited by more than 96% versus control values. After animals were killed at 3 months, injured vessel and graft segments were evaluated morphometrically. Although the response between animals was variable, average cross-sectional areas of neointima did not differ between the cilazapril-treated and control groups at sites of carotid endarterectomy (0.26 ± 0.12 versus 0.34 ± 0.17 mm^2, respectively; $p>0.5$), femoral artery ballooning (0.15 ± 0.08 versus 0.11 ± 0.01 mm^2; $p>0.5$), or at graft anastomoses (1.86 ± 0.50 versus 1.72 ± 0.50 mm^2; $p>0.5$). Thus, cilazapril did not reduce intimal thickening over 3 months in these primate arterial injury models. However, a possible beneficial effect of cilazapril, which might be apparent at earlier time points or with larger animal groups, cannot be excluded. (*Hypertension* 1991;18[suppl II]:II-70–II-76)

Restenosis due to smooth muscle cell proliferation is an important complication of interventional procedures involving arterial dilatation or reconstruction to improve blood flow in the management of symptomatic atherosclerotic vascular disease.[1,2] Attempts to modify this process pharmacologically have met with limited success.[3,4] Recently, it has been reported that inhibition of angiotensin converting enzyme (ACE) activity by administration of the oral ACE inhibitor cilazapril significantly reduced (by approximately 80%) the intimal proliferative response to carotid artery balloon catheter injury in the rat.[5] Although the mechanism whereby ACE inhibition affects smooth muscle cell growth in this model remains to be defined, the marked benefit observed implies that this approach could have important therapeutic implications for limiting the response to arterial injury in several clinical settings.

In the present report, the effects of cilazapril on the development of intimal proliferative lesions after mechanical injury to normal arteries were studied in a nonhuman primate model. Baboons were chosen as the experimental animal because of their primate vascular anatomy and because the primate hemostatic mechanism[6,7] and renin-angiotensin system[8–10] are similar to those in humans. Experimental arterial injury was produced by three different methods simulating therapeutic procedures performed in humans: 1) carotid artery endarterectomy, 2) surgical placement of synthetic vascular grafts, and 3) balloon catheter vessel dilatation. In each case, injury sites

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Supported by a grant from F. Hoffmann–La Roche Ltd., by research grants HL-31469 and HL-30946 from the National Heart, Lung, and Blood Institute, and in part by National Institutes of Health grant RR-00165 from the National Center for Research Resources to the Yerkes Regional Primate Research Center.

Address for correspondence: Stephen R. Hanson, PhD, Division of Hematology-Oncology, Drawer AJ, Emory University, Atlanta, GA 30322.

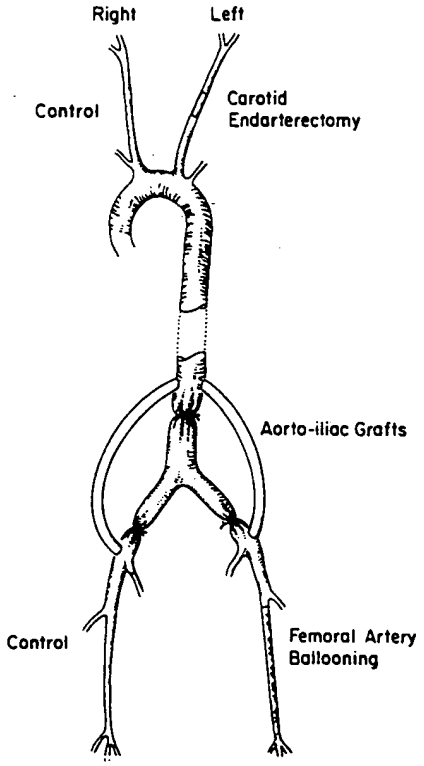

FIGURE 1. *Illustration of arterial injury models. Baboons underwent procedures for 1) balloon catheter injury of one superficial femoral artery, 2) endarterectomy of one carotid artery, and 3) surgical placement of bilateral aorto-iliac vascular grafts.*

were evaluated after 3 months by quantitative morphometric analysis with results for animals treated daily with cilazapril compared with results obtained in untreated control animals.

Methods

Twelve juvenile male baboons *(Papio cynocephalus)* aged 2–3 years and weighing 12–16 kg were used in these studies. Animals were quarantined and observed to be disease free for at least 90 days before use. All baboons were fed a normal diet; in two animals, serum cholesterol levels averaged 94–130 mg/dl. All procedures were approved by the Institutional Animal and Use Committee and were conducted in accordance with federal guidelines (*Guide for the Care and Use of Laboratory Animals,* National Institutes of Health Publication No. 86-23, 1985,. The Yerkes Center is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

All procedures were performed under sterile conditions with general halothane (in oxygen) anesthesia after induction with ketamine hydrochloride (20 mg/kg i.m.). Figure 1 illustrates the methods used to produce arterial injury. In all animals, the femoral artery ballooning and carotid artery endarterectomy procedures were performed concurrently. In nine of 10 animals (five treated and four controls), these procedures were performed approximately 2 weeks after the surgical placement of vascular grafts; in one animal (control), this sequence was reversed. Two additional control animals received vascular grafts only. After surgery, all animals were treated with antibiotics (Cefonicid, 25 mg/kg t.i.d. for 3 days). The analgesic used was buprenorphine (0.01 mg/kg t.i.d. for 3 days). All animals recovered uneventfully, without evidence of peripheral ischemia or neurological impairment.

Ten animals (five treated and five controls) underwent balloon catheter denudation of the superficial femoral artery according to a standard method.[11] After an incision performed over the medial aspect of the distal thigh, a side branch on the distal superficial femoral artery (near the takeoff of the popliteal artery) was isolated without exposing the proximal superficial femoral artery and controlled using vessel loops. A 3F Fogarty balloon catheter was passed through the branch to a distance of 10 cm (to the femoral bifurcation), inflated to a diameter of approximately 4 mm by filling with sterile saline, and withdrawn the length of the superficial femoral artery (to the insertion site) using a gentle twisting motion. A moderate but not strong resistance to the passage of the balloon was achieved in all cases. To ensure complete deendothelialization, this procedure was repeated three times with no instance of vessel rupture. This method also produces significant medial injury, with disruption of the internal elastic lamina (unpublished observations). After ballooning, the catheter was withdrawn, the side branch access vessel was ligated, and the incision site was closed. Only one artery in each animal was ballooned, with the contralateral superficial femoral artery serving as an uninjured control for morphometric comparisons.

Carotid endarterectomy procedures were performed in 10 animals (five treated and five controls) as described.[12] After a midline neck incision, one common carotid artery was dissected free of surrounding tissue from the aortic arch proximally to the bifurcation distally. Heparin (100 units/kg) was given intravenously, and the common carotid artery was occluded with atraumatic microsurgical vascular clamps placed at each end of the exposed vessel. The artery then was divided at a point 1 cm proximal to the distal clamp, and the proximal segment was everted over curved forceps to obtain exposure of the vessel intimal surface. The everted surface was held in place using 7-0 polypropylene stay sutures, and the endarterectomy was begun 1 cm from the divided end of the artery and continued for 1 cm. The endothelium, internal elastic lamina, and a portion of the media were removed using microvascular forceps and a surgical operating microscope under ×32 magnification. After the endarterectomy, the vessel was restored to its normal configuration, and an end-to-end anastomosis was accomplished using continuous 7-0 polypropylene sutures.

After endarterectomy procedures were performed in eight animals (three treated, five controls), platelet

TABLE 1. Effect of Cilazapril (20 mg/kg/day) in Baboons

Measurement	Therapy duration		
	Pre	1 Week	3 Weeks
ACE activity (units/ml)	59.2±6.5	1.8±0.7	2.0±0.8
ACE inhibition (%)	0	97.2±0.9	96.5±1.2

Values are mean±SEM. ACE, angiotensin converting enzyme.

deposition onto the endarterectomy sites was measured by scintillation camera imaging as described.[12,13] One hour before surgery, autologous platelets harvested from 100 ml whole blood and labeled with 1 mCi of Indium-oxine-111 (Amersham) were infused. Images of the neck were taken by acquiring both indium-111 energy peaks (172 and 247 keV) in 128×128 word mode using a General Electric 400T gamma camera (15% energy window with a high-energy collimator) coupled to a Medical Data Systems A^2 image processing system. Deposited (noncirculating) radioactivity at the injury site was determined by measuring total emissions (circulating plus deposited) at the endarterectomy site and subtracting the emissions from a region of the same size (1×2 cm) placed over the normal contralateral artery. Total platelet deposition (labeled plus unlabeled cells) was calculated as described.[12,13]

Bilateral aorto-iliac vascular grafts were placed as described by Clowes et al.[14-16] Using standard vascular techniques, expanded polytetrafluoroethylene (ePTFE) vascular grafts (Gore-Tex, W.L. Gore and Associates, Inc., Flagstaff, Ariz.) were inserted into the iliac circulation. Grafts were 4 mm i.d., 30 μm internodal distance, and 3–5 cm long. After a midline incision, grafts were placed bilaterally between the distal aorta and the common iliac artery (at the iliac bifurcation) using end-to-side anastomoses and continuous 7-0 polypropylene sutures. The distal aorta and common iliac artery then were ligated, with restoration of blood flow through the grafted segments.

Cilazapril (20 mg/kg once daily) was administered orally (by gavage) beginning 1 week before the first surgical procedure and was continued throughout the study interval. This dosage was tolerated well, although two animals given cilazapril in a greater amount (40 mg/kg/day) showed some evidence of hepatic toxicity (increased plasma liver enzymes). During surgery, the mean arterial blood pressure of cilazapril-treated baboons was reduced minimally (by less than 10 mm Hg) as compared with untreated animals. At 1 week and 3 weeks after the first surgical procedure, blood samples were taken for determination of plasma ACE activity using an assay previously described.[17] As shown in Table 1, at both time points, plasma ACE activity was inhibited by greater than 96% versus control values. This level of ACE inhibition also should represent a minimum value, because samples for assay were taken approximately 24 hours after dosing, that is, immediately before drug administration on the following day.

TABLE 2. Patency Rates After Arterial Injury

Group	Carotid artery endarterectomy	Femoral artery ballooning	Aorto-iliac grafts
Controls	5/5	5/5	8/14
Cilazapril	3/5	5/5	7/10

Animals were killed after approximately 3 months (grafts, 93±4 days; carotid and femoral arteries, 83±8 days, ±1 SD) by in vivo pressure–perfusion fixation.[12,14] Briefly, after ketamine analepsis and halothane anesthesia were achieved, both internal jugular veins and both femoral veins were cannulated for subsequent exit drainage. Thirty minutes before perfusion, animals were given heparin (300 units/kg) and Evans blue dye (50 mg/kg) intravenously. Mechanical ventilation was initiated and a median sternotomy performed. The pericardium was incised, and a 10-gauge needle (connected to the perfusion delivery system) was inserted into the left ventricle. Initial perfusion was with 2–3 l of 0.1 M phosphate buffered saline, followed by perfusion for approximately 15 minutes of 8–10 l perfusate consisting of 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffered saline, pH 7.4. Perfusion flow rates were adjusted to maintain the intra-arterial pressure at 80–100 mm Hg as determined by a pressure monitor placed in the thoracic aorta. Both carotid arteries, femoral arteries, and graft specimens were removed en bloc and placed for 90 minutes in fixative consisting of 2% paraformaldehyde, 0.1% glutaraldehyde, and 0.1 M sodium cacodylate, pH 7.4, at 4°C. The proximal half of each normal and injured femoral and carotid artery (divided at the endarterectomy midpoint) and one graft of each pair placed in individual animals were sent to F. Hoffmann–La Roche Ltd (Basel, Switzerland) after being transferred to a shipping buffer consisting of 0.1 M sodium cacodylate, 7% sucrose, and 0.05 M NH_4Cl, pH 7.4, at 4°C. Specimens subsequently were embedded in paraffin or epoxy resin for cross sectioning, morphometry, and histological evaluation.[11,12,14,18,19] Cross sections were taken perpendicular to the vessel long axis. Vascular grafts embedded in paraffin were sectioned at the midpoint between the heel and toe of each anastomosis, at a distance 1–2 mm from the heel of each anastomosis, and at 5-mm intervals along the entire graft length. Morphometric measurements were performed using a Zeiss photoscope coupled with a computer-assisted display system (Thomas Optical, Columbus, Ga.) and image analysis software (OPTIMAS, Bioscan, Inc., Edmonds, Wash.).

Comparisons between groups were made using the Student's t test (two-tailed) for paired and unpaired data. Unless otherwise stated, values are given as mean±1 SEM.

Results

The patency rates of explanted artery and graft segments are given in Table 2 and averaged: 100%

TABLE 3. Femoral Artery Injury: Ballooning

Group	Balloon injury			Noninjured
	Area of neointima (mm^2)	Area of media (mm^2)	Neointima/media (×100%)	Area of media (mm^2)
Control (5)	0.11±0.01	0.82±0.13	14.2±2.0	1.01±0.22
Cilazapril (5)	0.15±0.08	1.04±0.16	13.9±4.9	0.85±0.16
	$p>0.5$	$p>0.3$	$p>0.5$	$p>0.5$

Values are mean±SEM. Numbers in parentheses are number of arteries.

(femoral arteries), 80% (carotid arteries), and 63% (vascular grafts). Cilazapril treatment had no apparent effect on these outcomes.

Balloon Catheter Injury of Femoral Arteries

The results of morphometric analysis of balloon-injured and normal superficial femoral arteries for the treated and control groups are given in Table 3. Stained sections are shown in Figures 2A and 2B. There was significant variability in the area of neointima that formed after balloon catheter injury, with values ranging 0.09–0.13 mm^2 in the control group and 0.0–0.45 mm^2 in the cilazapril-treated animals. Total medial areas averaged approximately 1 mm^2 and were comparable in injured and uninjured vessels. The ratio of the area of neointima to media (×100%) averaged approximately 14%. There was no difference ($p>0.3$) between treated and untreated groups with respect to these measurements. Similarly, the analysis at Hoffmann–La Roche of different vessel segments from the same animals indicated that neointimal areas were equivalent in the control and treated groups (0.16±0.04 versus 0.16±0.03 mm^2, respectively; $p>0.5$).

Carotid Artery Endarterectomy

Indium-111 platelet imaging of carotid endarterectomy sites 1 hour postoperatively showed that the injured regions in treated and control animals were comparably thrombogenic acutely, accumulating $4.4\pm0.8\times10^8$ and $4.5\pm1.8\times10^8$ platelets, respectively ($p>0.5$).

The morphometric analyses of carotid artery explants are given in Table 4. Histological sections are illustrated in Figures 2C and 2D. The area of neointima was variable in both control animals (0–0.8 mm^2, $n=5$) and in cilazapril-treated baboons (0.12–0.49 mm^2, $n=3$). Medial areas averaged approximately 1.5 mm^2 in both injured and uninjured vessels. The ratio of neointima to media (×100%) was not different between control and treated groups (20.5±10.1% versus 13.9±6.8%, respectively; $p>0.5$). Endarterectomy segments from the same animals analyzed at Hoffmann–La Roche also indicated that areas of neointima were not different between control and treated groups (0.30±0.18 versus 0.46±0.36 mm^2, respectively; $p>0.5$).

Aortoiliac Vascular Grafts

All explanted vascular grafts (except the longest 5-cm graft) were completely healed after 3 months as indicated by the exclusion of Evans blue dye. The thickness of neointima was measured as a function of distance from the anastomoses of grafts taken from untreated control animals (10 anastomoses from five grafts) and from cilazapril-treated animals (six anastomoses from three grafts). Results are shown in Figure 3. The graft morphology is illustrated in Figures 2E and 2F. Neointimal thickening was maximal at the anastomoses, averaging 0.2–0.3 mm and declining to approximately 0.1 mm in the midgraft region. Grafts from control and treated baboons were not different with respect to intimal thickness ($p>0.5$ at all graft locations). Four additional grafts from cilazapril-treated animals evaluated at Hoffmann–La Roche also exhibited neointima having a midgraft thickness in this range (0.21±0.09 mm; Figure 3).

Similarly, total neointima area was greatest near the graft anastomoses (Figure 4), varying from 1.2 to 4.7 mm^2, and declined toward the graft midpoint. Four grafts from treated animals evaluated at Hoffmann–La Roche exhibited neointimal areas averaging 2.72±0.66 mm^2 in the midgraft region. These results were consistent with previous observations by Clowes et al[14–16] with untreated animals studied in the same graft model (Figure 4).

Discussion

Cilazapril did not reduce intimal proliferative lesion formation in baboons in the three models of intimal hyperplasia investigated. Because the number of animals studied was small and the response between animals was variable, these results do not exclude a possible limited benefit of cilazapril. Nonetheless, the ineffectiveness of therapy was a consistent finding in the three models studied. Although the reasons for this result are unclear in light of the reported marked benefit of cilazapril therapy in a rat model of carotid artery balloon injury,[5] it would seem unlikely that these differences were simply related to the drug administration regimen or to the techniques used for producing vascular injury. Because the previous study in rats indicated that maximum inhibition of neointima formation was achieved by inhibition of ACE activity both before arterial injury and contin-

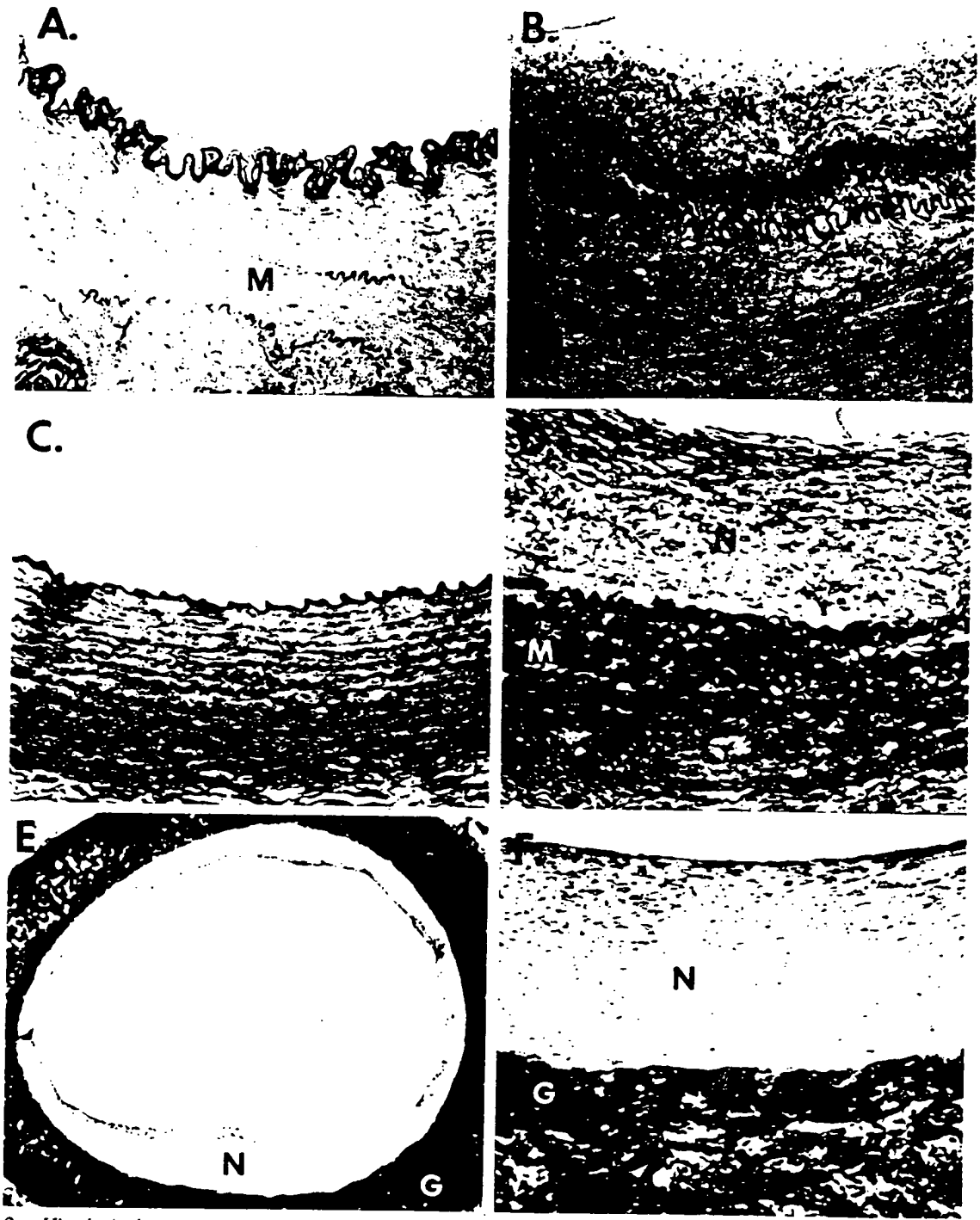

FIGURE 2. *Histological cross sections of arterial injury sites from untreated animals.* Panels A *and* B: *Uninjured (control) femoral artery (panel A) is compared with balloon catheter–injured vessel (panel B) showing significant intimal thickening after 3 months. Epoxy resin–embedded sections stained with toluidine blue and basic fuchsin.* Panels C *and* D: *Uninjured carotid artery (panel C) and vessel taken 3 months after carotid endarterectomy (panel D). Paraffin embedding and elastic stain (Verhoeff–van Gieson).* Panels E *and* F: *Whole graft cross section showing typical eccentric lesion (panel E) and neointima overlying graft matrix (panel F). Paraffin embedding with hematoxylin and eosin stain. M, media; N, neointima; G, graft matrix (polytetrafluoroethylene). Magnifications: panels A and B, ×184; panels D–F, ×120; panel E, ×17.6.*

uously during the response of the vascular wall,[5] therapy in the present study was initiated 1 week before injury and continued throughout the study interval of 3 months. Also, the dose administered (20 mg/kg/day) was twice that administered in the previous study reporting a benefit. Under these conditions, plasma ACE activity was effectively abolished (Table 1).

TABLE 4. Carotid Artery Injury: Endarterectomy

Group	Endarterectomy: Area of neointima (mm^2)	Endarterectomy: Area of media (mm^2)	Endarterectomy: Neointima/media (×100%)	Noninjured: Area of media (mm^2)
Control (5)	0.34±0.17	1.57±0.09	20.5±10.1	1.53±0.11
Cilazapril (3)	0.26±0.12	1.89±0.07	13.9±6.8	1.50±0.05
	$p>0.5$	$p>0.1$	$p>0.5$	$p>0.5$

Values are mean±SEM. Numbers in parentheses are number of arteries.

The three types of arterial injury studied differ in several important respects. For example, sites of carotid endarterectomy and arterial graft placement are both acutely thrombogenic as demonstrated by indium-111 platelet imaging,[12,13] and equivalent platelet uptake was found after carotid endarterectomy in control and cilazapril-treated animals (see "Results"). However, despite vigorous balloon injury, we have not observed significant platelet accumulation on vessels injured by this method, probably because scintillation camera imaging is insensitive for detecting partial platelet monolayers or small platelet thrombi (unpublished observations). Thus, although the possible contribution to intimal thickening of components derived from the hemostatic mechanism (e.g., thrombin, platelet-derived growth factor) may differ in these models, lesion formation was significant in each case and unaffected by cilazapril.

In addition, there are important differences in healing mechanisms after different types of injury and between species. After carotid artery ballooning in the rat, a maximal proliferative response requires medial injury as well as denudation,[20] with total smooth muscle cell number reaching a maximum at 2 weeks.[21] Endothelium is regenerated from the ends of the denuded vessel, but segments may remain chronically denuded of endothelium and weakly reactive toward platelets for periods up to 1 year.[21,22] Cilazapril inhibits the proliferative response of smooth cells in the rat for at least 8 weeks[23] and perhaps up to 6 months (J.S.P., unpublished data). In contrast, after endarterectomy or ballooning of the baboon carotid artery, endothelialization is complete within 1 month, partly because of the proliferation and migration of cells derived from small vessels penetrating the artery lumen (Reference 12 and unpublished observations). Although the time course of cell ingrowth and proliferation has not been systematically studied after femoral artery balloon injury in the baboon, a qualitatively similar mechanism may apply, because after 3 months, even relatively long vessel segments appeared completely healed, as evidenced by the exclusion of Evans blue dye. Thus, in the femoral artery balloon injury and carotid endarterectomy models, it may be that an early benefit of cilazapril was not observed with our specimens because they were evaluated late after complete healing had already occurred; that is, rapid reendothelialization may have limited the proliferative response.

The time course of cellular responses to implanted vascular grafts has been studied extensively in the baboon.[14–16] After placement of clinical (Gore-Tex)

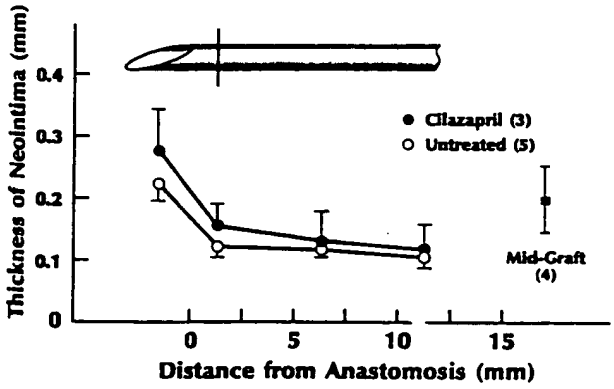

FIGURE 3. *Thickness of neointima measured from the anastomoses of vascular grafts after 3 months. Intima was thickest at the graft anastomoses and declined toward the graft midsection. Results with three grafts (six anastomoses) from cilazapril-treated animals (●) and with five grafts (10 anastomoses) from control animals (○) were equivalent and comparable to results with four additional grafts from treated animals evaluated at the graft midpoint only (■). Values are mean±SEM.*

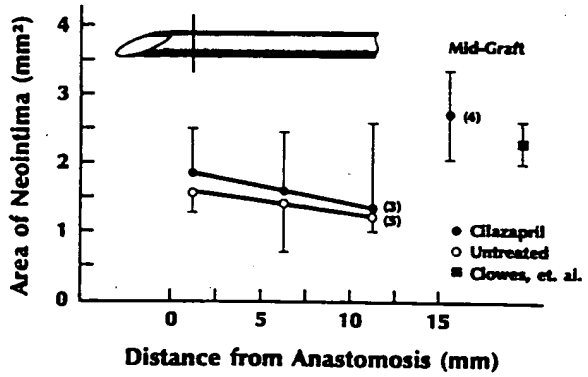

FIGURE 4. *Area of graft neointima after 3 months. The area of neointima measured from graft anastomoses was equivalent between five control animals (○, 10 anastomoses) and three animals treated with cilazapril (●, six anastomoses). Results with four additional grafts from treated animals evaluated the graft midregion only (●, midgraft) were comparable to results previously reported by Clowes et al[14] using the same graft model (■). Values are mean±SEM.*

vascular gr[redacted] endothelial and smooth muscle cells derived from the cut ends of adjacent artery migrate along the graft luminal surface to form neointima. Smooth muscle cell mass increases up to 3 months, remaining constant thereafter[15]; hence, this end point also was used in the present study. Capillaries do not penetrate the graft midsection, which may remain nonendothelialized with continued accumulation of circulating platelets and other blood elements. Intimal cross-sectional area always is greatest at the graft anastomoses, where smooth muscle cells continue to proliferate despite an overlying layer of confluent endothelium.[14-16] The results of the present morphometric study were in agreement with these earlier observations. The ineffectiveness of cilazapril in this setting (Figures 3 and 4) may be due in part to these differences in cell proliferation between grafts and injured arteries.[14]

The lack of benefit of cilazapril in baboons as opposed to rats also may be due to species-specific effects. In the rat studies,[5,23] it has been suggested that ACE inhibition specifically leads to reduced intimal thickening, a conclusion supported by observations that captopril, an ACE inhibitor that is chemically distinct from cilazapril, is comparably efficacious in that model.[5] This mechanism would require inhibition of local (tissue-associated) converting enzyme systems,[5,8,24,25] which might vary considerably between species with regard to the actions of specific antagonists. Thus, there may be important differences in tissue penetration, metabolism, and clearance of cilazapril between the two species, rat and baboon.[26] Measurements of the inhibition of plasma converting enzyme may not be indicative of the actual degree of inhibition of the tissue converting enzyme systems. These considerations, together with the results of the present study, suggest that species-related differences may be important in the evaluation of agents designed to limit the proliferative response to arterial injury.

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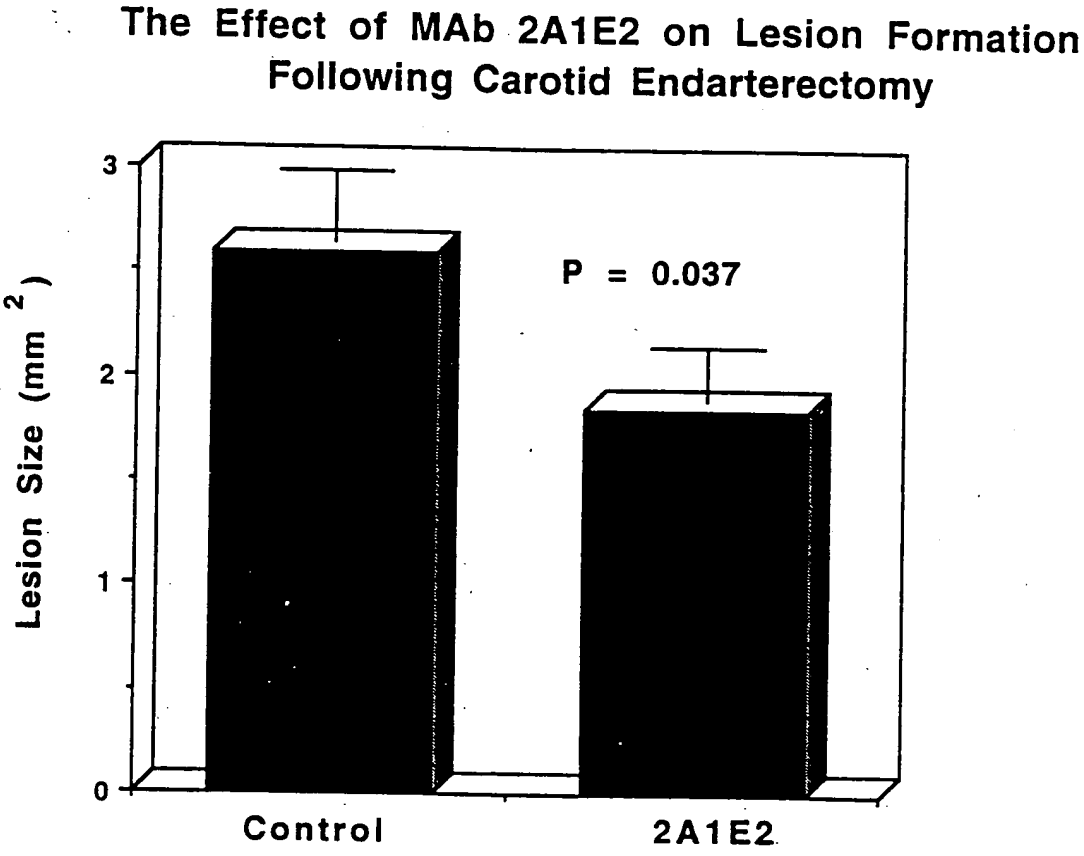
KEY WORDS • angiotensin converting enzyme inhibitors • hyperplasia • primate studies

FIGURE 1



ATTACHMENT C

FIGURE 2